ORIGINAL ARTICLE

Nitric oxide and reactive species are modulated in the polyphenol-induced ductus arteriosus constriction in pregnant sheep

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ABSTRACT

Objective Because we have previously demonstrated the relation between polyphenol-rich foods (PRF) consumption and ductus arteriosus constriction, in this work, pregnant sheep were submitted to oral PRF intake for 14 days to understand how this process occurs. Fetal Doppler echocardiography, oxidative and inflammatory biomarkers and total polyphenol excretion were evaluated.

Results The high polyphenol intake induced ductus arteriosus constriction by 71.6% increase in systolic ($P = 0.001$) and 57.8% in diastolic velocities ($P = 0.002$), and 18.9% decrease in pulsatility index ($P = 0.033$), along with 1.7-fold increase in total polyphenol excretion, 2.3-fold decrease in inflammatory mediator nitric oxide and following redox status changes (mean ± standard deviation): higher protein carbonyls (1.09 ± 0.09 and 1.49 ± 0.31), catalase (0.69 ± 0.39 and 1.44 ± 0.33) and glutathione peroxidase (37.23 ± 11.19 and 62.96 ± 15.03) in addition to lower lipid damage (17.22 ± 2.05 and 12.53 ± 2.11) and nonprotein thiols (0.11 ± 0.04 and 0.04 ± 0.01) found before and after treatment, respectively. Ductal parameters correlated to NO_x, catalase, glutathione peroxidase and protein carbonyl.

Conclusion Our results highlight the need to reduce maternal PRF intake in late pregnancy to prevent fetal duct constriction through NO-mediated vasoconstrictive action of polyphenols. © 2014 John Wiley & Sons, Ltd.

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INTRODUCTION

Polyphenols are phytochemicals well studied for contributing to the prevention of cancer, cardiovascular and neurodegenerative diseases,[1–4] and present in a variety of food sources, especially vegetables, fruits, tea (*Camellia sinensis*), cocoa and nuts. Most health benefits ascribed to polyphenols may be related to their prominent antioxidant and anti-inflammatory effects, once oxidative damage and inflammation are usually present in chronic and degenerative diseases.[5–7] Despite the scarce evidence about the safety of polyphenol consumption, these compounds are normally well tolerated in usual amounts, presenting few adverse effects that may either occur after ingestion of higher doses[8–10] or interactions with other drugs.[11,12]

During pregnancy, a vascular duct called ductus arteriosus (DA) remains patent to allow the blood flow to circulate into lower fetal portions. Ductal patency is controlled by local production of prostaglandins and nitric oxide, and as gestation proceeds, the duct becomes less sensitive to dilating prostaglandins and more sensitive to constricting influences, for example, arterial oxygen tension.[13] The DA closes physiologically after birth with the onset of pulmonary circulation.[14] However, premature DA constriction in the third trimester of pregnancy may lead to pulmonary hypertension in the newborn or fetal death. Premature DA constriction has been reported after nonsteroidal anti-inflammatory drugs (NSAID) or glucocorticoids administration[15]; thus, these drugs are usually avoided in late pregnancy.

Evidences indicate that maternal consumption of prostaglandin synthetase inhibitors leads to DA sensibilization, which may cause its constriction.[13] Our group has previously reported that

maternal consumption of polyphenol-rich foods (PRF) interferes with ductal flow in human fetuses, probably by a polyphenol-induced anti-inflammatory effect,[16,17] and also that restriction of PRF consumption was able to reverse ductal constriction.[18] In this context, the present study aimed to investigate the interrelations of fetal duct dynamics, oxidative damage and inflammatory markers after PRF administration to pregnant sheep in late pregnancy.

METHODS

Study design

The study included six adult female Corriedale sheep (90–100 kg) in late pregnancy (gestational age of >120 days), which corresponds to the third trimester of pregnancy. Animals were fed for 2 weeks with standardized amount of PRF (basal intake + 3100 mg/day). The ewes received the usual oral diets, which consist of alfafa, milled corn and mineral salt that were supplemented with PRF selected by a nutritionist and incorporated to the food (dried tomatoes, dried apples, milled and dried green tea leaves and raw soy grains), and the total polyphenol levels in PRF supplement were quantified by the spectrophotometric Folin–Ciocalteu reaction. Before the 14-day period, the animals were adapted for 7 days in the experimentation site. Each animal before treatment has its own control in relation to the posttreatment group. Animals were kept and handled in a proper location in the University Veterinary Hospital (HCV/UFRGS), and water was available *ad libitum* for consumption according to the guidelines of the local committee. All animals were identified by arbitrary codes present in tags attached to their ears, which were unknown to the analysts during Doppler or biochemical analyses. This study was approved by the Ethics Committee in Research (IC/FUC) under No. UP3888/06.

Collection of biological samples

Samples before treatment or basals as well as samples after treatment were collected before and after the 14 days of experiment, respectively. At both moments, venous blood samples were collected by venipuncture into Vacutainer® (BD Diagnostics, Plymouth, UK) tubes with EDTA, sodium heparin and without anticoagulants. Urine samples were collected at both moments in sterile and light-protected recipients, and stored at −80 °C until analysis. Blood and urine collection were performed by experienced veterinaries.

Fetal Doppler echocardiography

Echocardiograms were obtained using two-dimensional Doppler color flow imaging with convex transducers 7 or 5 MHz and/or a sectorial-phased array of 3.5 or 5 MHz with the General Electric Logic 4 system, with 2D pulsed and continuous Doppler and color flow mapping capability. The ewes were put in position, laying on their backs, and secured by trained animal handlers from the Veterinary Medicine School of University of Rio Grande do Sul. Their abdominal surfaces were shaved, and the same gel commonly utilized for human echocardiography was used as interface with the transducer. Possible technical difficulties were prevented by extensive previous training during a period of several months with pregnant ewes. All the examinations were performed with the help of a veterinarian echocardiographist, who participated in the studies giving his technical insights.

In 2D echocardiography, the DA was imaged both in a modified three vessels view and in the long axis view of the ductal arch, and Doppler velocities were measured by positioning the sample volume in the descending aortic end of the DA, with a maximal insonation angle of 20°. The typical DA flow waveform was obtained. Systolic and diastolic peak velocities were determined manually, utilizing the electronic caliper of the equipment and considering the mean of three measurements in fetal apnea. Pulsatility index of ductal flow was electronically calculated by the machine after manual tracing of the complete waveform, including systolic, diastolic and mean velocities, being the calculation on the basis of the ratio (systolic peak velocity − diastolic peak velocity/mean velocity).

The ratio between the right and left ventricular dimensions was obtained on a four-chamber view in late diastole to assess right ventricular repercussion. Increases in mean ductal velocities greater than 20% after exposure were considered signs of ductal constriction, as previously established by our group.[17] All examinations were performed by the same pediatric cardiologists with experience in fetal echocardiography. The presence of ductal flow turbulence, tricuspid and/or pulmonary regurgitation and leftward interventricular septal bulging were searched.

Total polyphenol urinary excretion

Quantification of total polyphenols (TP) in urine was performed as previously reported.[19] Briefly, urine samples stored at −80 °C were thawed for 3 h on ice bath and centrifuged at 4 °C for 10 min. Samples were diluted and acidified, and then a cleanup procedure by solid phase extraction with Waters Oasis® MAX 30-mg cartridges (Milford, MA, USA) was performed. Extracted eluates reacted with Folin–Ciocalteu reagent 2 M and 20% sodium carbonate in 96-well microplates and incubated for 1 h in the dark. Absorbances were read at 765 nm. Urinary creatinine was determined to correct TP excretion by spectrophotometry using commercial kits (Doles reagents, Goiânia, GO, Brazil). TP excretion was expressed as milligram gallic acid equivalents (GAE) per gram creatinine.

Lipid peroxidation

Lipid peroxidation was evaluated by the measurement of thiobarbituric acid reactive substances (TBARS), in which plasma-EDTA was processed and absorbances were measured at 535 nm as previously described.[20] TBARS levels were estimated as micromoles malondialdehyde equivalents per liter using tetramethoxypropane as standard.

3-Nitrotyrosine (3-NT) levels

Plasma 3-nitrotyrosine was assessed by noncompetitive ELISA method.[21] Total proteins were measured by the Bradford method, and plasma was diluted to 2 mg protein per milliliter and incubated in Maxisorb multiwallplates (Nunc Immuno 96 Microwell™ Maxisorp) overnight at 4 °C in the dark. Polyclonal anti-nitrotyrosine (Millipore) and monoclonal goat anti-rabbit

IgG, HRP-conjugate (Millipore), were used as primary and secondary antibodies, respectively. Absorbance was measured at 492 nm in triplicates, and results were expressed as picomoles per milligram protein.

Protein carbonyl (PCO) levels

Protein carbonyls were determined by noncompetitive ELISA method following some modifications.[22] Total plasma proteins were measured by Bradford. Plasma was diluted with PBS buffer (4 mg protein mL^{-1}), derivatized with 2,4-dinitro-phenylhydrazine and incubated in Maxisorb multiwallplates (Nunc Immuno 96 Microwell™ Maxisorp) overnight at 4 °C in the dark. PCOs were detected using dinitrophenyl rabbit IgG-antiserum (Sigma, Deisenhofen, Germany) as primary antibody and monoclonal anti-rabbit immunoglobulin G peroxidase conjugate (Sigma) as secondary antibody. Absorbances were measured at 492 nm in triplicates, and results were expressed as nanomoles per milligram protein.

Reduced nonprotein thiol groups

Determination of reduced nonprotein thiols in erythrocytes was performed by spectrophotometry.[23] Red blood cells were hemolyzed by Triton X-100 and precipitated with 20% trichloroacetic acid (w/v) after 10 min. After centrifugation, 10 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) was added to supernatants. DTNB, or Ellman's reagent, reacts with reduced thiols producing mixed disulfides (Ellman's derivates) and anion 5-thio-2-nitrobenzoate, which is quantified by its strong visible absorbance at 412 nm as an indirect measure of reduced thiols. Results were expressed as micromoles per liter red blood cells.

Enzymatic antioxidants

Catalase (CAT) activity was determined as previously described.[24] Enzymatic activity was evaluated by monitoring the rate of H_2O_2 decomposition by CAT at 240 nm during 5 min at 37 °C. CAT activity was expressed as K CAT per milligram protein. Glutathione peroxidase (GPx) was measured by a method in which absorbances were monitored at 340 nm at 37 °C.[25] GPx activity was expressed as micromoles NADPH per minute per milligram protein. Furthermore, glutathione S-transferase (GST) activity was determined at 340 nm using 1-chloro-2.4-dinitrobenzene (CDNB) as substrate and 0.15 M glutathione (GSH).[26] GST activity was expressed as nanomoles CDNB conjugated with GSH per minute per milligram protein.

Prostaglandin E2 (PGE2) levels

Prostaglandin E2 levels were measured by commercial kit on the basis of a competitive enzyme immunoassay method (Enzo Life Sciences, Farmingdale, NY, USA), following the manufacturer's instructions. Absorbance was determined at 405 nm, and results were expressed as picograms per milliliter.

Nitrite/nitrates (NO_x) ratio

Serum nitrites/nitrates were determined according to the modified Griess method.[27] First, nitrates present in samples were reduced to nitrites after reaction with vanadium (III) chloride (VCl_3) 0.08%. Then a mixture of sulfanilamide 2%, N-(1-naphthyl)ethylenediamine (NED) 0.2% and orthophosphoric acid in distillated/deionized water (Griess reagent) was added to the samples. Sulfanilamide reacts with nitrites in the samples to form a diazonium salt that reacts with NED to produce a purple-azo-dye product, which is measured at 540 nm in Cobas Mira® (Roche Diagnostics, Basel, Switzerland). Results were expressed as micromoles per liter.

Statistical analysis

Data were analyzed utilizing IBM SPSS Statistics software (version 19.0), and all study variables were tested for normality by the Shapiro–Wilk test. Comparisons between groups were performed by paired Student's *t*-test or Wilcoxon signed rank test, and results are expressed as mean ± standard deviation or median (interquartile range), according to the distribution of variables. Correlation test used was Pearson's correlation coefficient or Spearman's rank. Significant differences were considered when $P \leq 0.05$.

RESULTS

In order to investigate the flow dynamics in the fetal DA, fetal hearts were analyzed by Doppler echocardiography. Analysis of echocardiographical parameters showed significant increases in systolic (SV) and diastolic velocities (DV) (Figure 1a), and decrease in pulsatility index (PI) (Figure 1b) in the animals after 14 days of dietary intervention versus basals. Percentual differences between before treatment and after treatment, respectively, were 71.6% for SV (0.75 ± 0.15 and 1.28 ± 0.26 m s^{-1}, $P = 0.001$), 57.8% for DV (0.18 ± 0.02 and 0.29 ± 0.04 m s^{-1}, $P = 0.002$) and 18.9% for PI (2.49 ± 0.18 and 2.02 ± 0.34, $P = 0.033$). Besides, echocardiographical images indicated a constriction of the DA in the PRF-treated animals (Figure 1d) in comparison with basals (Figure 1c), compatible with the previous parameters that showed signs of ductal constriction.

Total polyphenol levels excreted in urine from the pregnant sheep after PRF intake were significantly increased in the 14-day-treated group compared with that of basal (Figure 2a).

As depicted in Table 1, oxidative damages were evaluated by biomarkers of lipid and protein damages. Lipid peroxidation as estimated by TBARS showed a reduction after 14 days compared with that of the basal state. Protein damage was investigated by PCO and 3-NT. Our results indicate significant PCO increase in animals after 14 days of treatment, and no significant difference was found in 3-NT levels ($P > 0.05$).

Erythrocyte levels of reduced nonprotein thiols were decreased in the treated group, comparing basal with 14 days (Table 1). Considering the enzymatic antioxidant systems, GPx and catalase activities were significantly increased after 14-day treatments, whereas no significant changes were observed in GST activity.

Serum inflammatory biomarkers investigated in the studied animals revealed that PRF treatment induced a decrease in NO levels after 14 days compared with basals (Figure 2b). Serum PGE2 showed no significant differences (data not shown).

Figure 3 shows negative correlations between NO_x levels and GPx ($r = -0.755$, $P = 0.004$) and also between NO_x and CAT ($r = -0.812$, $P = 0.001$). In addition, we have observed a positive correlation between lipid peroxidation and NO_x ($r = 0.748$, $P = 0.005$) and a negative correlation between lipid peroxidation and the TP excretion, $r = -0.622$, $P = 0.03$ (Figure 4).

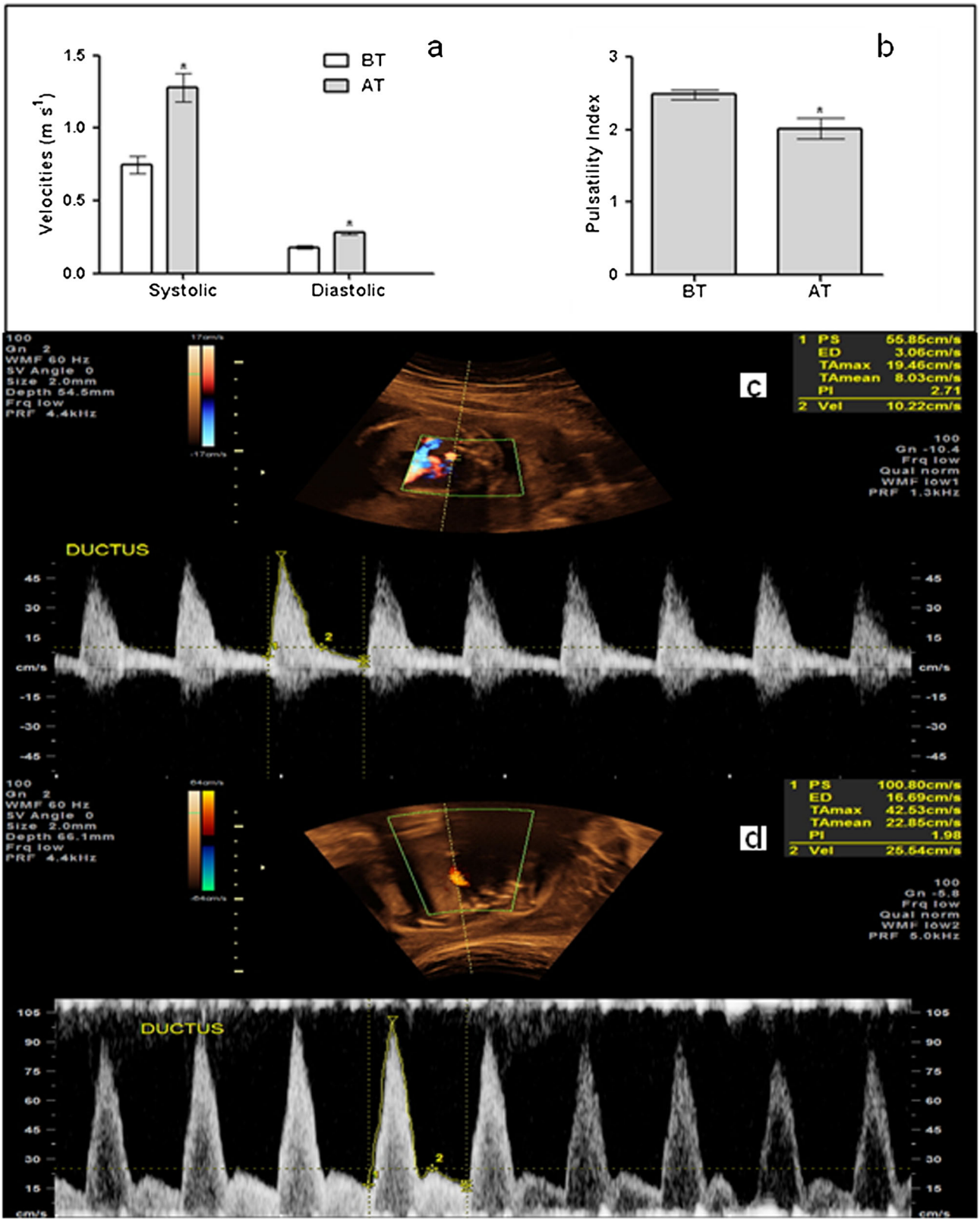

Figure 1 Induction of ductus arteriosus constriction by fetal Doppler echocardiography analysis in sheep after high polyphenol consumption during the third trimester of pregnancy. Bars (a) and (b) respectively represent systolic/diastolic velocities and the pulsatility index. Echocardiographical images of the ductus arteriosus before (c) and after treatment (d). *Significant differences in relation to basal ($P < 0.05$, $n = 6$, paired Student's *t*-test). BT, before treatment/basal; AT, after treatment

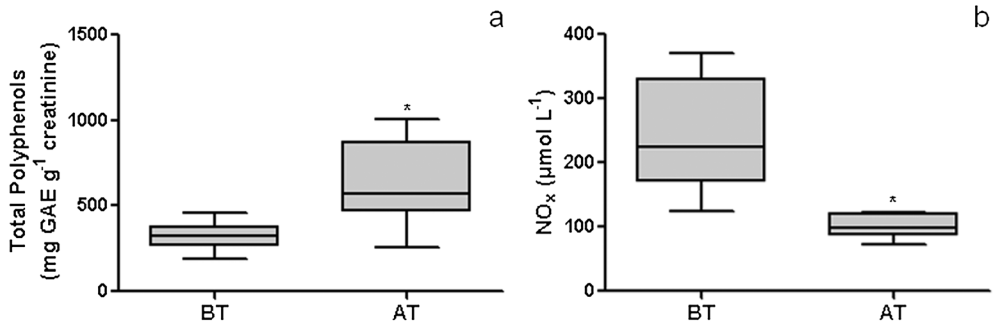

Figure 2 Effects of polyphenol-rich foods on circulating nitric oxide levels and excreted total polyphenols. (a) Total polyphenol urinary excretion in pregnant sheep increased after dietary supplementation; (b) serum nitric oxide levels decreased in pregnant sheep submitted to high polyphenol ingestion in the third trimester of pregnancy. *$P=0.028$ for both significant differences in relation to basal ($n=6$, Wilcoxon signed ranks test). BT, before treatment/basal; AT, after treatment

Table 1 Oxidative stress biomarkers in pregnant sheep submitted to high polyphenol ingestion in the third trimester of pregnancy

Biomarkers	Before treatment/basal	After treatment
TBARS (μmol L^{-1})	17.22 ± 2.05	12.53 ± 2.11**
3-Nitrotyrosine (ρmol mg^{-1} protein)	5.34 ± 0.91	6.48 ± 2.02
Protein carbonyl (nmol mg^{-1} protein)	1.09 ± 0.09	1.49 ± 0.31*
Nonprotein thiols (μmol mL^{-1} red blood cells)	0.11 ± 0.04	0.04 ± 0.01**
Glutathione peroxidase (μmol NADPH min^{-1} mg^{-1} protein)	37.23 ± 11.19	62.96 ± 15.03**
Glutathione S-transferase (nmol CDNB-GSH min^{-1} mg^{-1} protein)	2.04 ± 1.03	1.38 ± 0.46
CAT (K CAT mg^{-1} protein)	0.69 ± 0.39	1.44 ± 0.33*

Results are shown as mean ± standard deviation. TBARS, thiobarbituric acid reactive substances; CDNB, 1-chloro-2.4-dinitrobenzene; GSH, glutathione; CAT, catalase. *$P<0.05$ and **$P<0.01$ indicate significant differences in relation to basal (paired Student's *t*test).

Ductal constriction parameters presented significant correlations between each other. A positive correlation was found between SV and DV ($r=0.686$, $P=0.028$), and a negative correlation between SV and IP ($r=-0.712$, $P=0.021$) was observed. We also found that echocardiographical parameters were associated to biomarkers of protein and lipid oxidative damage. PCO correlated to SV ($r=0.629$, $P=0.028$), DV ($r=0.905$, $P=0.0001$) and to IP ($r=-0.772$, $P=0.003$). However, TBARS presented inverse correlations to the echocardiographical parameters, that is, TBARS versus SV ($r=-0.746$, $P=0.013$), TBARS versus DV ($r=-0.825$, $P=0.003$) and TBARS versus IP ($r=0.660$, $P=0.038$).

Antioxidant enzymes also presented correlations with ductal parameters, for example, CAT versus SV ($r=0.672$, $P=0.033$) and GPx versus IP ($r=-0.629$, $P=0.05$). In addition, significant correlations between echocardiographical measures and NO_x were also observed (Figure 5).

DISCUSSION

In the present study, a high polyphenol intake in female sheep during late pregnancy induced alterations in the ductal flow dynamics characteristic of fetal duct constriction. A previous study from our group showed that green tea extract, which also presents significant contents of polyphenols, when orally administered to pregnant sheep was able to induce similar echocardiographical alterations observed in the present study[17]; however, the underlying mechanisms responsible for the DA constriction were not evaluated.

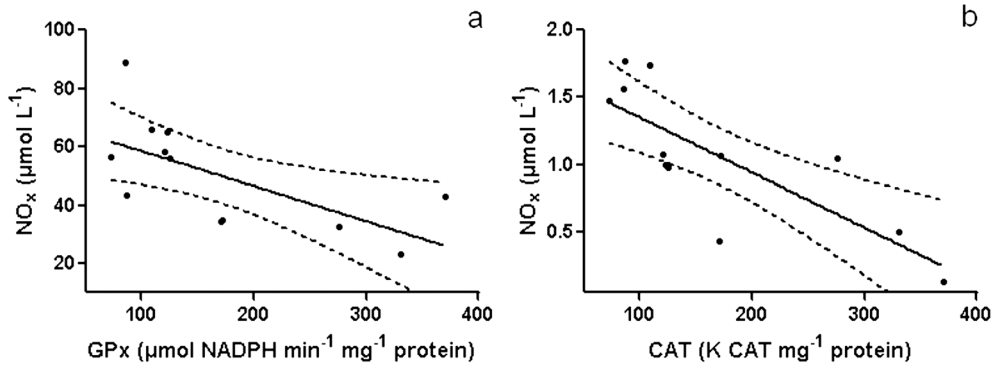

Figure 3 Associations between nitric oxide and antioxidant enzymes. (a) GPx versus NO_x ($r=-0.755$, $P=0.004$, $n=12$) and (b) CAT versus NO_x ($r=-0.812$, $P=0.001$, $n=12$). Spearman's rank was used for both statistical correlations

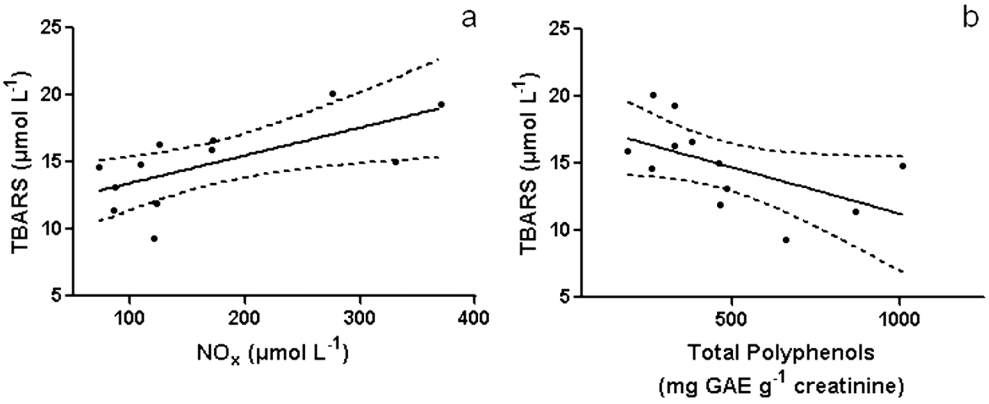

Figure 4 Associations of lipid damage with (a) nitric oxide levels ($r = 0.748$, $P = 0.005$, $n = 12$) and (b) total polyphenol excretion ($r = -0.622$, $P = 0.030$, $n = 12$). Spearman's rank was used for both statistical correlations

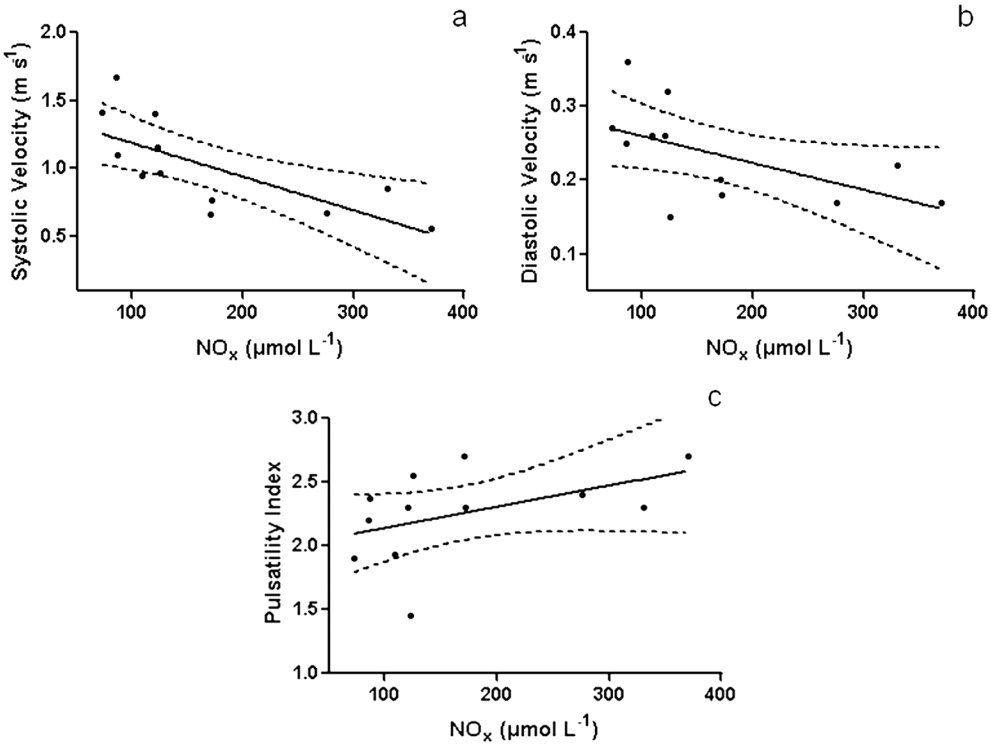

Figure 5 Associations among nitric oxide levels and fetal Doppler echocardiographical parameters. (a) Nitric oxide versus SV ($r = -0.853$, $P = 0.0004$, $n = 12$), (b) nitric oxide versus DV ($r = -0.705$, $P = 0.010$, $n = 12$) and (c) nitric oxide versus PI ($r = 0.599$, $P = 0.039$, $n = 12$). Spearman's rank was used for all statistical correlations

The increased TP excretion indicates that PRF ingestion was effective once polyphenols were absorbed and eliminated in urine, representing an important biomarker of TP intake. Subsequently, mechanisms of oxidative stress and inflammatory biomarkers were measured. Lipid peroxidation is usually represented by the by-product malondialdehyde and other aldehydes produced in the reactive oxygen species (ROS)-induced peroxidation of membrane lipids and estimated as TBARS. Polyphenols are markedly associated to antioxidant properties in several studies,[5,28,29] and the lower TBARS found in our study are in accordance with these previous data. Lipid peroxidation during pregnancy has also been described especially with the approximation of labor due to higher ROS production.[30]

Furthermore, PCOs were increased, despite the decrease in lipid damage also observed, suggesting a partial antioxidant effect in relation to protein damage, once polyphenols were able to protect against lipid damage. 3-NT was not altered, which could be ascribed to the short 3-NT plasma half-life (1–2 h) and the transient 3-NT alterations as a consequence of protein degradation, repair or clearance.[31,32]

Regarding the antioxidant defenses, a mobilization is expected as an attempt to prevent oxidative damages to biomolecules.[33,34] Herein, the decreased levels of reduced

nonprotein thiols suggest that despite the polyphenol consumption, the overproduction of reactive species typical of late pregnancy induced a mobilization of nonprotein thiols to preserve tissues from oxidative damage. In addition, catalase and GPx were activated, clearly to scavenge the ROS overproduction.[30,35,36] Taken together, the GPx, TBARS and nonprotein thiols results indicate the involvement of the GPx and GSH system to scavenge lipid peroxides generated in order to protect against damage to lipid membranes.

Polyphenols, which are well recognized exogenous antioxidants,[28,29,37] possibly acted synergistically with endogenous antioxidant defenses against ROS unbalance but were still associated to echocardiographical alterations in fetuses' hearts. Protein damage suggests that ROS overproduction in pregnancy could not be completely scavenged by endogenous antioxidants and some extent of oxidative stress could have contributed to the premature cardiovascular alterations in the fetuses, in addition to the NO-induced vasoconstrictive effect. Indeed, the previous reports back up the involvement of ROS overproduction in DA constriction by activating different signaling pathways, such as inhibiting voltage-gated potassium channels and activating Rho-kinase signaling, leading to smooth muscle contraction[38–40] and also affecting the mitochondrial redox state.[36]

Nitric oxide (NO) is a reactive nitrogen species (RNS) generated from the endothelial nitric oxide synthase and acting as a signaling molecule, especially leading to vasodilatation, neurotransmission and inflammation.[41] The NO radical has a very short serum half-life and reacts with circulating reactive species to produce different stable inorganic metabolites, such as nitrites and nitrates[42]; thus, nitrite/nitrate (NO_x) levels are often used as a measure of NO production. Polyphenols present anti-inflammatory activities,[43,44] and nitric oxide production generates free radicals by nitric oxide synthase[45]; therefore, NO inhibition by polyphenols is also congruent with their antioxidant activities.

Moreover, lower NO levels in conjunction with echocardiographical analysis in PRF-treated sheep indicate that polyphenols were responsible for vascular constriction. Some studies have reported that NO is responsible for maintaining DA patency, which is inverted in late pregnancy, as the fetal duct becomes physiologically less sensitive to NO, leading to the physiological DA closure.[13,14] Our results indicate that polyphenols are able to accelerate this process when consumed in late pregnancy, leading to premature DA constriction. Similarly, a clinical trial from Keller and coworkers[46] also showed that premature newborns with patent ductus arteriosus developed DA constriction after treatment with both NOS inhibitor L-NMMA and indomethacin, showing the role of NO-mediated vasoconstriction in the induction of ductal constriction.[46]

In our study, no significant difference in PGE2 levels could be observed; however, further prostaglandin biosynthesis biomarkers should be evaluated, for example, additional prostaglandins, isoprostanes and arachidonic acid in serum or cyclooxygenase (COX) isoforms in blood lymphocytes.[47,48] Recently, Chen and coworkers[49] revealed isoprostanes as novel biomarkers associated to oxidative stress and responsible to promote DA constriction in mice. Exposures to 8-iso-PGF2α and 8-Iso-PGE2 isoprostanes induced fetal DA constriction in preterm pregnant mice by binding thromboxane A2 receptors.[49] Isoprostanes are produced from oxidative damage to lipids, especially arachidonic acid, whereas prostaglandins are released from COX activities.[50] Therefore, isoprostane synthesis seems more promising than prostaglandins in this process considering the concomitant involvement of oxidative stress and NO inhibition, which could explain the absence of significant PGE2 alterations, requiring further confirmation.

The studies available on the DA dynamics are basically based on the effect of anti-inflammatory drugs (NSAIDs or glucocorticoids) either in inducing premature DA constriction[51–53] or in patent ductus arteriosus cases, in which the pharmacological closure of the DA is searched.[54] Therefore, few studies have been conducted with a premature DA constriction without any relation to NSAIDs intake,[16,18,55] which are anti-inflammatory agents, whereas polyphenols in general present simultaneous anti-inflammatory and antioxidant effects, as shown in our results.

In previous studies from our group, Zielinsky and coworkers demonstrated that the intake of PRF during late pregnancy was able to induce premature DA constriction in human fetuses[16] and recently reported the reversal of ductal constriction after pregnant women were instructed not to consume foods with high polyphenol contents.[18] These findings were corroborated by Kapadia and coworkers, who associated the prenatal DA closure with maternal ingestion of a juice blend containing anthocyanins and proanthocyanidins.[55]

In addition to the increase in protein damage by protein carbonyl levels (Table 1), we interestingly observed that oxidative modification to proteins was associated to ductal alterations by the high correlations between PCO and SV, PCO and DV, and PCO and IP, indicating that ductal constriction was influenced by protein damage. On the other hand, lipid peroxidation was decreased in pregnant sheep after PRF intake, and echocardiographical parameters (SV, DV and IP) were inversely associated to lipid damage, suggesting that lipid damage did not contribute to ductal constriction and that polyphenols were able to promote some protection against lipid damage.

Considering that polyphenols contributed to diminish lipid damage (Figure 4b) and that polyphenols also decreased NO (Figure 2), it could be explained that the PRF-treated animals presenting lower plasma NO also demonstrate less lipid damage, as evidenced in Figure 4a, because of the polyphenol-induced vasoconstriction and protection against lipid damage, once both activities were exerted by polyphenols. Also, NO and its stable products nitrites/nitrates are RNS that could lead to lipid damage,[56] so lower NO could be responsible for the decreased lipid peroxidation.

Importantly, the correlations between NO and both systolic and diastolic velocities as well as the correlation between pulsatility index and NO directly associate the decreased NO and the development of fetal duct constriction in the treated animals.

In addition to RNS, the increasing oxygen tension in fetuses during late pregnancy near birth is responsible for the physiological DA constriction,[13,38] consistent with the increasing ROS reported in the approximation of labor.[30] The correlations of SV versus CAT and IP versus GPx also confirm the involvement

of antioxidant enzymes in the premature ductal constriction. The antioxidant and vasoconstrictive behaviors of polyphenols, observed by the negative correlation of NO_x to both GPx and CAT, demonstrated that the increase in these antioxidants was associated to NO inhibition. NO has been reported to reversibly bind and inhibit catalase,[57] explaining the strong negative correlation observed.

CONCLUSION

Animal models are currently necessary to elucidate the mechanisms involved in polyphenol-induced ductal constriction. The present study not only reports that PRF consumption during late pregnancy induces DA constriction but this is to our knowledge the first work to indicate the ability of polyphenols to inhibit NO thus leading to vasoconstriction responsible for the mechanism of DA constriction, in addition to modulating oxidative pathways. Finally, our findings stress the need to reduce maternal polyphenol consumption in late pregnancy to circumvent the development of premature ductal constriction.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Polyphenol-rich foods have been associated to premature ductal constriction when consumed in the third trimester of pregnancy.

WHAT DOES THIS STUDY ADD?

- Polyphenols revealed to induce vasoconstriction of the ductus arteriosus by inhibiting nitric oxide vasodilatation. Consumption of polyphenol-rich foods in late pregnancy showed partial antioxidant effect by contributing to reduce lipid peroxidation. In contrast, NO-mediated ductal vasoconstriction could also be associated to oxidative damage to proteins.

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